

have measured CD and absorption spectra of ba_3 -type cytochrome *c* oxidase in the fully reduced ($\text{b}^{2+}\text{a}_3^{2+}$), fully oxidized ($\text{b}^{3+}\text{a}_3^{3+}$), reduced CO-bound ($\text{b}^{2+}\text{a}_3^{2+}\text{-CO}$) and mixed-valence, CN-bound ($\text{b}^{3+}\text{a}_3^{2+}\text{-CN}$) states. CD spectra in the Soret absorption band of both enzymes are very non-conservative, with the area under positive lobe of the spectra strongly prevailing over the area under the negative part. Knowing the crystal structure of the enzyme, we attempted at modeling the CD spectra of the fully reduced and fully oxidized forms of bovine CcO using classical polarizability theory. With this approach, we can explain the non-conservative character of the CD spectra in the Soret band and reproduce reasonably well the magnitudes and lineshapes of the CD signals. The analysis suggests that optical activity of the enzyme in the Soret band originates in the interaction of the hemes with each other as well as with the aromatic amino-acid residues around.

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11P.5 Role of membrane potential on the control of cytochrome *c* oxidase over respiration in intact hepatoma HepG2 cells

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Metabolic control analysis (MCA) [1] has been largely applied to the analysis of oxidative phosphorylation, in order to investigate the control exerted by each individual reaction step on the whole pathway [2]. Using this approach, the control exerted by the electron transport chain complexes was shown to be higher in experimental systems closer to *in vivo* conditions than in mitochondria. To study the effect of the mitochondrial transmembrane proton electrochemical gradient ($\Delta\mu_{\text{H}^+}$) on the control of respiration by cytochrome *c* oxidase (CcOX) in intact cells, we applied MCA to mitochondrial respiration of HepG2 cells. Both the overall O_2 consumption and specific CcOX activity of actively phosphorylating cells were progressively inhibited by cyanide titration under conditions in which the electrical ($\Delta\psi$) and/or the chemical (ΔpH) component of $\Delta\mu_{\text{H}^+}$ was selectively modulated by addition of ionophores. Under endogenous conditions, i.e., in the absence of ionophores, CcOX displayed a high control coefficient value, thus representing an important site of regulation of mitochondrial oxidative phosphorylation. A high CcOX control coefficient value was also measured in the presence of nigericin, when $\Delta\psi$ is maximal, and in the presence of nigericin and valinomycin, when $\Delta\mu_{\text{H}^+}$ is abolished. On the contrary and interestingly CcOX displayed a markedly lower control coefficient in the presence valinomycin converting $\Delta\psi$ into ΔpH . These results show that CcOX activity and its control over oxidative phosphorylation critically depend on $\Delta\psi$ in actively phosphorylating cells [3].

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11P.6 Comparison of the recombinant wildtype with the ATCC wildtype of cytochrome *c* oxidase from *Paracoccus denitrificans*

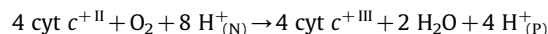
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The cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans* consists of four subunits and is the terminal member of the respiratory chain. It pumps four protons from the negative (N) to the positive (P) side of the membrane and catalyses the reduction of molecular oxygen to water using four more protons.



This process takes place in the protein's active binuclear center, housing a haem a_3 and a copper ion (Cu_B). In order to compare variants and the wild type (WT) in a more controlled way a recombinant WT was constructed carrying the SU I gene on a low copy number plasmid. Comparing the rec WT to the ATCC WT no differences were expected, yet a significant increase in the reactivity towards hydrogen peroxide was found for the rec WT. This observation raises the question: Why do these two differently expressed wild type forms of the enzyme vary in this reactivity? Presumably a somehow misplaced Cu_B leads to an increase in hydrogen peroxide reactivity. To test this hypothesis we used a His-tagged variant in order to complex copper during assembly of the protein. Therefore the His-tag could be used as a copper source for copper inserting chaperones during assembly. Additionally extra copies of both bacterial chaperones *ctaG* and *surflc* required for metal insertion into SU I were cloned on the same plasmid along with the SU I gene to compensate the slight overproduction of SU I from the plasmid. We constructed variants H276R and H276K, and tested them for their activity. These variants presumably lack Cu_B but maintain the overall electrostatic properties. Additionally the variants H276D and H276E were expressed to potentially incorporate a Fe(III) ion instead of the Cu (II) ion. The negative charge for the side chain was chosen to maintain the overall electrostatic properties in this site when incorporating a ferric iron. The results of metal content measurements as well as the results of activity assays will be presented.

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11P.7 Structural studies of catalytic intermediates in cytochrome *c* oxidase

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Cytochrome *c* oxidase (Cyt cO) is the final electron acceptor in the electron-transport chain in the inner membrane of mitochondria and bacteria. This enzyme catalyzes the reduction of molecular oxygen to water and energetically couples this reaction to proton transfer across the biological membrane. In this project we use X-ray crystallography together with time-resolved spectroscopic techniques to study the specific intermediates of cytochrome *c* oxidase formed during catalysis. By solving the three dimensional crystal structures of these short-lived intermediates we aim to obtain a better understanding for how Cyt cO accomplishes the coupling between electron- and proton transfers during the reduction of molecular oxygen to water. In our attempts to trap these intermediates we use structural variants of the enzyme that have been shown to stop the catalytic reaction at specific steps in the reaction cycle. A second approach we use is soaking

crystals of the wild-type enzyme with reagents such that specific states of the enzyme are formed. So far crystals of various qualities have been obtained using the different approaches and datasets from crystals diffracting to high resolution are being processed.

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11P.8 Novel structure–function relationships of plant alternative oxidase

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Alternative oxidase (AOX) is a terminal oxidase of mitochondrial respiratory chain, which catalyzes oxidation of ubiquinol and reduction of molecular oxygen in a coupling manner. Although no data from X-ray crystallography is available for structural analysis to date, structure–function relationships of the enzyme have been well characterized with respect to its highly-conserved elements for catalytic and regulatory mechanisms. On the other hand, such relationships involving less-conserved ones, which may confer unique enzymatic characteristics to respective AOX isozymes, still remain uncharacterized. Here, we show that novel structure–function relationships exist in *Arum concinatum* AOX1a (AcoAOX1a) and that they involve at least three less-conserved structural elements, all of which have not been reported with functional significance except for E83. E83K was previously reported to deprive AOX activity in chimera mutants carrying segmented structures derived from AcoAOX1a and its inactive homologue AcoAOX1b. Nevertheless, our recent data from site-directed mutagenesis of E83K in wild-type AcoAOX1a did not show any significant alteration in the activity, suggesting that interactive effects exist between E83 and the other structural elements held in AcoAOX1a. Careful interpretation of these data predicts some candidate amino acid residues of AcoAOX1a that coordinately function with E83 in retaining its catalytic activity, and furthermore, leads to new proposal for a hypothetical model of their intramolecular interactions. Of particular interest is that these candidate residues lie globally in the primary structure, implying that structural complexity exists beyond a currently supported model of AOX. In this poster, we present our recent findings about structure–function relationships of AcoAOX1a and discuss possible structural interactions involving their responsive residues.

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11P.9 Peculiarities of cyanide binding to *ba*₃-type cytochrome oxidase from a thermophilic bacterium *Thermus thermophilus*

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Cyanide complexes of the reduced heme *a*₃ in cytochrome *ba*₃ and mitochondrial *aa*₃-type cytochrome oxidase are similar spectroscopically. However, affinity of the reduced heme *a*₃²⁺ for cyanide may be strikingly different for the two enzymes. In dithionite-reduced bovine oxidase, *K*_d for cyanide binding with heme *a*₃²⁺ is 0.4–0.5 mM, in agreement with the data in literature [1]. With *ba*₃

cytochrome oxidase, the affinity of ferrous heme *a*₃ for cyanide is found to depend on the reduction procedure. In the presence of mild reductants (e.g., ascorbate + diaminodurene) or when reduced by dithionite without redox mediators, heme *a*₃²⁺ binding with cyanide is extremely tight: *K*_d value for cyanide complex of cytochrome *ba*₃ of the enzyme does not exceed 10^{−8} M. The high affinity of cytochrome *ba*₃ for cyanide is associated mainly with extremely low rate of ligand dissociation (*k*_{off} < 10^{−6} s^{−1}), while the rate of binding (*k*_{on} about 10² M^{−1} s^{−1}) is similar to that observed for the mitochondrial cytochrome oxidase [2]. However, if the *ba*₃-type oxidase is reduced by dithionite in the presence of redox mediators which greatly accelerate interaction of the enzyme with the reductant, the *K*_d for cyanide binding increases to about 0.5 mM, close to the value observed with the mitochondrial oxidase. It is proposed that cyanide affinity for the ferrous heme *a*₃ in *ba*₃ oxidase is controlled by the redox state of Cu_B. Binding of a second CN[−] to the oxidized Cu_B may lock heme *a*₃²⁺-bound cyanide in the binuclear site.

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11P.10 Nitric oxide dependent electron transfer and proton uptake in bacterial nitric oxide reductase

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Nitric oxide reductase (NOR) from *Paracoccus denitrificans* is a deviant heme copper oxidase (HCuO) and catalyzes the two electron reduction of NO to N₂O (2NO + 2H⁺ + 2e[−] → N₂O + H₂O). Under aerobic conditions the four electron reduction of O₂ to H₂O is catalyzed as a side reaction. In contrast to observations in other HCuOs, neither of the reactions contributes to the proton motive force. NOR purifies as a two subunit complex (NorBC) harboring four redox-active co-factors. A high-spin heme *b*₃ and Fe_B, an anti-ferromagnetically coupled non-heme iron analogous to Cu_B in HCuOs, comprise the binuclear active site in NorB. A low-spin heme *b* in NorC accepts electrons from NorC, a membrane-anchored cytochrome *c*. Using the flow-flash technique we have studied the [NO] dependency of four electron reduced NOR capable of two turnovers: Initially, NO binds to heme *b*₃ with a time constant in the μs range at 1.5 mM NO. This binding is concentration-dependent, which is inconsistent with a previously proposed obligatory binding of NO to Fe_B prior to ligating to heme *b*₃. In the first turnover, NO is reduced by two electrons from the active site. Further oxidation of NOR involves biphasic electron transfer from the low-spin hemes with rates of 50 and 3 s^{−1} at pH 7.5 and 1.5 mM NO. At lower (0.075 mM) [NO] these phases accelerate 3–4-fold. This acceleration is more pronounced (17-fold) at pH 6 due to proton uptake from bulk solution, which is coupled to oxidation of the low-spin hemes. We could demonstrate that substrate inhibition, observed in steady-state measurements, occurs already during the oxidation of the fully reduced enzyme. NO binds to its inhibitory site before electrons can redistribute to the active site from the low-spin hemes *b* and *c*.

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